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TECHNICAL MANUSCRIPT 570

EFFECT OF EDTA  
ON THE INFECTIVITY OF VEE VIRUS

Ralph F. Wachter  
Jo Ann F. Comer  
James W. Johnson

JANUARY 1970

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EFFECT OF EDTA ON THE INFECTIVITY OF VEE VIRUS

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BIOLOGICAL SCIENCES LABORATORIES

Project 1B061102B7 1A

January 1970

# ABSTRACT

Although a number of plant viruses are inactivated by ethylenediamine-tetraacetic acid (EDTA), we have found that this chelating agent stabilizes Venezuelan equine encephalitis (VEE) virus and other group A arboviruses very effectively over the temperature range 4 to 50 C. In the presence of  $5 \times 10^{-3}$  M EDTA, the infectivity of partially purified VEE virus was maintained in demineralized water with no significant decrease for 3 days at 37 C, 2 to 4 weeks at 25 C, and 3 to 10 months at 4 C; at these temperatures control suspensions without EDTA lost 99.99% of their infectivity in 1 day, 2 to 4 days, and 10 to 30 days, respectively. These results were obtained with water or phosphate buffer suspensions only at the optimal pH range of 6.8 to 7.5. This degree of stabilization is comparable to that reported for poliovirus with 1 M  $Mg^{++}$ . Although the presence of EDTA is required for consistently successful phenol extraction of infectious RNA (IRNA) from partially purified VEE virus, thermal stabilization of IRNA by EDTA was not observed. Results obtained with density gradient centrifugation and other techniques using  $C^{14}$ -labeled EDTA suggest that EDTA does not form a stable complex with VEE virus; chelation of trace amounts of inactivating metal ions may constitute the mode of action.

CONTENTS

Abstract . . . . .	2
I. INTRODUCTION . . . . .	5
II. MATERIALS AND METHODS . . . . .	5
III. RESULTS . . . . .	6
IV. DISCUSSION . . . . .	14
Literature Cited . . . . .	15
Distribution List . . . . .	17
DD Form 1473 . . . . .	19

FIGURES

1. Stabilization of Partially Purified VEE Virus by EDTA at 50 C . .	7
2. Stabilization of Partially Purified VEE virus by EDTA at 37 C . .	7
3. Stabilization of Partially Purified VEE Virus by EDTA at 25 C . .	8
4. Stabilization of Partially Purified VEE Virus by EDTA at 4 C . .	8
5. Effect of pH on the Stabilization of VEE Virus by EDTA at 37 C .	10
6. Sucrose Gradient Centrifugation of C <sup>14</sup> -EDTA and VEE Virus Mixture . . . . .	11

TABLES

1. Effect of Calcium Ion on EDTA Stabilization of VEE Virus . . . .	12
2. Effect of Cobalt Ion on EDTA Stabilization of EEE Virus . . . .	13
3. Effect of Manganese Ion on EDTA Stabilization of VEE Virus . . .	13

## I. INTRODUCTION\*

For the successful extraction of infectious ribonucleic acid (IRNA) by the phenol method from partially purified Venezuelan equine encephalitis (VEE) or Eastern equine encephalitis (EEE) viruses, the presence of ethylenediaminetetraacetic acid (EDTA) was usually essential. Reports in the literature indicate that other investigators also found this to be true. EDTA and other chelating agents have been shown to inactivate a number of plant viruses.<sup>1-4</sup> White et al.<sup>5</sup> reported the inactivation of some lipid-containing animal viruses by metal chelates of substituted 1,10-phenanthrolines.

Investigations were initiated to determine the effect of EDTA on the infectivity of VEE virus and other arboviruses. This paper reports that VEE, EEE, and Sindbis viruses, members of the group A arboviruses, rather than being inactivated, are stabilized exceedingly well by EDTA.

## II. MATERIALS AND METHODS

Partially purified suspensions of the Trinidad-donkey-brain strain of VEE virus, the Louisiana strain of EEE virus, and the Egyptian AR 339 strain of Sindbis virus were prepared for use in these tests from culture fluids of chick fibroblast or L cell monolayers by one cycle of low- and high-speed centrifugation (Spinco 30 rotor, 11,700 x g for 10 minutes; 73,350 x g for 1.5 to 3 hours). Viral sediments of the high-speed centrifugation were rinsed with small volumes of demineralized water or buffer, then resuspended to original volume in either water or 0.02 M phosphate buffer. For most tests, equal volumes of virus suspension and EDTA solution were mixed and distributed in 2-ml volumes to sterile, 10-ml, polyethylene tubes with tight-fitting caps. The disodium salt of EDTA, which we have used routinely, in water solution at  $10^{-2}$  M has a pH of about 4.5. When EDTA was dissolved in phosphate buffer solution of pH 7.8, a pH value of 6.8 was obtained. Later, when water solutions of EDTA were investigated, pH was adjusted to values in the range 6.8 to 7.5 with NaOH. Stability tests with the viruses were conducted at 4, 25 (room temperature), 37, and 50 C.

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IRNA employed in these studies was extracted from partially purified suspensions of VEE virus with hot phenol according to the procedure of Fraenkel-Conrat<sup>6</sup> and was dissolved in either demineralized water or 0.02 M phosphate buffer, pH 7.4.

Infectivity of virus samples by the amniotic route of inoculation was determined by titration in 14-day embryonated eggs. Infectivity of IRNA samples was assayed by plaque formation in monolayer cultures of chick fibroblast cells that were pretreated with hypertonic NaCl.<sup>7</sup>

### III. RESULTS

The effect of EDTA, at a concentration of  $5 \times 10^{-3}$  M, on the stability of VEE virus in buffer suspension at 50 C is shown in Figure 1. The compound had a pronounced effect on viral stability. The same results were obtained when the viral pellet from high-speed centrifugation was or was not rinsed to remove residual culture fluid.

Figure 2 indicates the marked effect of EDTA on the stability of partially purified VEE virus at 37 C. In the absence of EDTA, virus in phosphate buffer lost 99.99% of its infectivity in 24 hours. In the presence of  $5 \times 10^{-3}$  M EDTA, no infectivity was lost in 3 days. This degree of stabilization is comparable to that reported by Wallis and Melnick<sup>8</sup> for the stabilization of poliovirus by 1 M  $Mg^{++}$  ion. In this experiment the pH was 6.8; as will be shown later, pH is a very important factor in obtaining maximum effectiveness of stabilization.

In  $5 \times 10^{-3}$  M EDTA, the initial infectivity titers of partially purified VEE virus suspensions have been maintained at 25 C for periods of 2 to 4 weeks, as shown by the data in Figure 3. After 5 weeks, virus samples still possessed titers of  $>10^6$  egg LD<sub>50</sub>/ml.

To evaluate the stabilizing effect of EDTA at 4 C, partially purified VEE virus was stored in triplicate in phosphate buffer in the presence or absence of  $5 \times 10^{-3}$  M EDTA. The results of two tests performed at different times are shown in Figure 4. In experiment A, samples maintained infectivity with no significant decrease for about 3 months, and at the end of 6 months the titer had decreased only about 1 log<sub>10</sub>. In experiment B, original infectivity was maintained for 2 weeks before it started to decrease at a relatively slow rate; after 18 months, 4.5 log<sub>10</sub> of infectious virus were still present. The reason for the difference in the two tests is not known, although it can be seen that the control virus of the experiment A lost infectivity more slowly than that of experiment B.

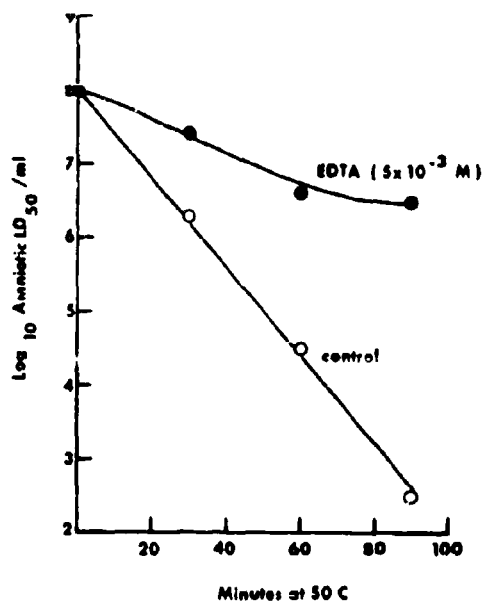


FIGURE 1. Stabilization of Partially Purified VEE Virus by EDTA at 50 C.

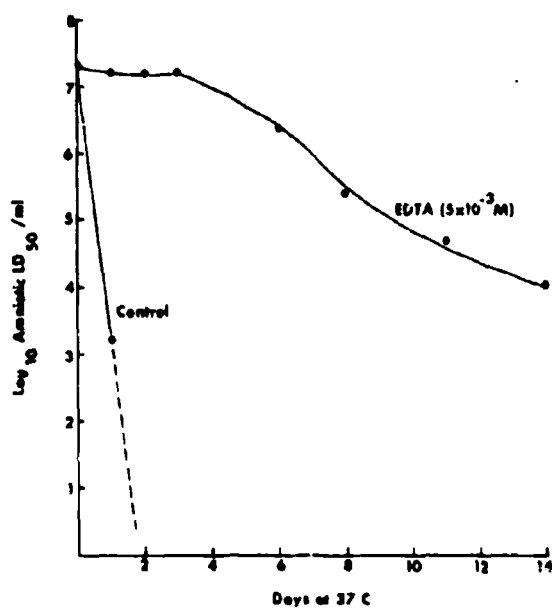


FIGURE 2. Stabilization of Partially Purified VEE Virus by EDTA at 37 C.

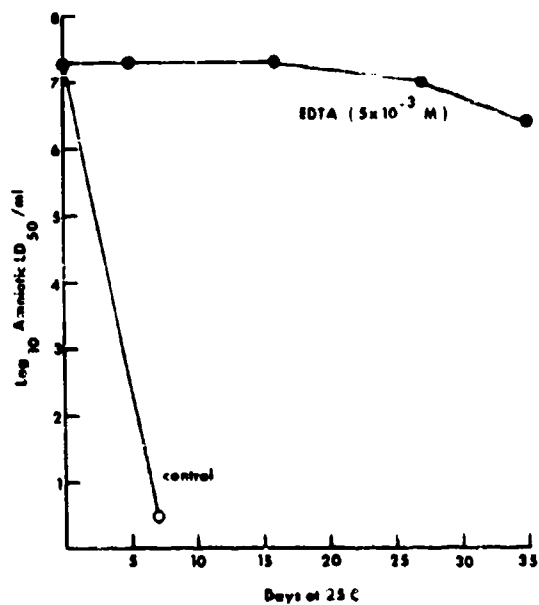


FIGURE 3. Stabilization of Partially Purified VEE Virus by EDTA at 25 C.

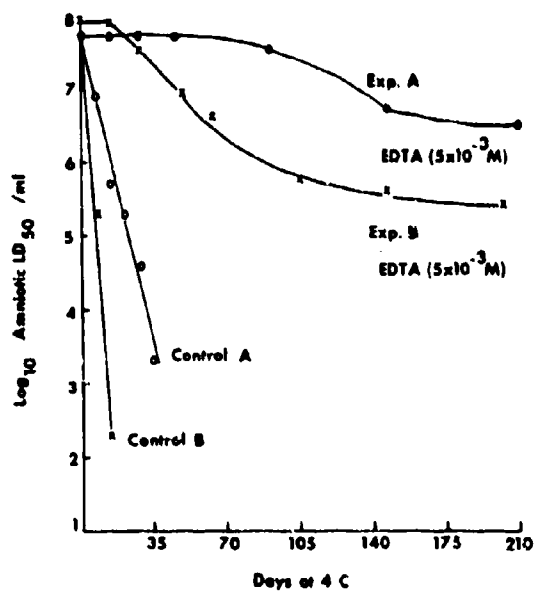


FIGURE 4. Stabilization of Partially Purified VEE Virus by EDTA at 4 C.

In a later test at 4 C, VEE virus suspended in a water solution of EDTA lost infectivity even more slowly, with no significant change in titer in 10 months.

The importance of pH in achieving maximum stabilization with EDTA is indicated by the data in Figure 5. Duplicate samples of partially purified VEE virus in  $5 \times 10^{-3}$  M EDTA in phosphate buffer were adjusted to pH values of 6.0, 7.0, and 8.0 and placed at 37 C. Much better virus stability was observed at pH 7 than at pH 6 or 8. Although VEE virus normally is much less stable at pH 6 than at pH 8, in the presence of EDTA the stability of the virus was the same at these pH levels. In this test, control samples in phosphate buffer lost 4  $\log_{10}$  or more of infectivity in 1 day, compared with less than 1  $\log_{10}$  for treated samples at pH 6 and 8; therefore, even under the less favorable pH conditions EDTA exerted a pronounced stabilizing effect.

Studies were also performed using preparations of the IRNA of VEE virus with and without added EDTA. In these experiments over a temperature range of 4 to 70 C, no significant differences were observed in the rate of loss of infectivity whether EDTA was present or absent. In considering these results, it should be noted that EDTA was always added to the partially purified virus preparation from which the IRNA was extracted, so that control samples in the stability tests possibly could contain low levels of EDTA in complex with the IRNA.

To determine whether EDTA does or does not bind to VEE virus or to IRNA,  $C^{14}$ -labeled EDTA was employed in sucrose gradient centrifugation experiments with both the virus and the nucleic acid. The procedure employed with the virus was as follows. To 40 ml of a partially purified VEE virus suspension was added 0.2 ml of  $C^{14}$ -labeled EDTA (10  $\mu$ C/ml). Five milliliters of this suspension were layered on a sucrose gradient (17 to 32%) in a 30-ml tube. After centrifugation for 3.5 hours at 25,000 rpm in a Spinco SW-25 rotor, 2-ml samples were collected through the bottom of the tube and assayed for infectivity and radioactivity. A portion of each sample was treated with trichloroacetic acid to precipitate any virus and/or nonviral protein. Precipitates were then assayed for radioactivity. Distribution of infectivity and radioactivity in the gradient is illustrated in Figure 6. Considering the nearly total lack of radioactivity in the trichloroacetic acid precipitate of fractions containing the peak of viral infectivity, it seemed unlikely that EDTA existed in complex with the virus particle.

This same type of density gradient experiment, using IRNA and labeled EDTA, gave essentially the same result: there was no indication that EDTA was associated with the IRNA.

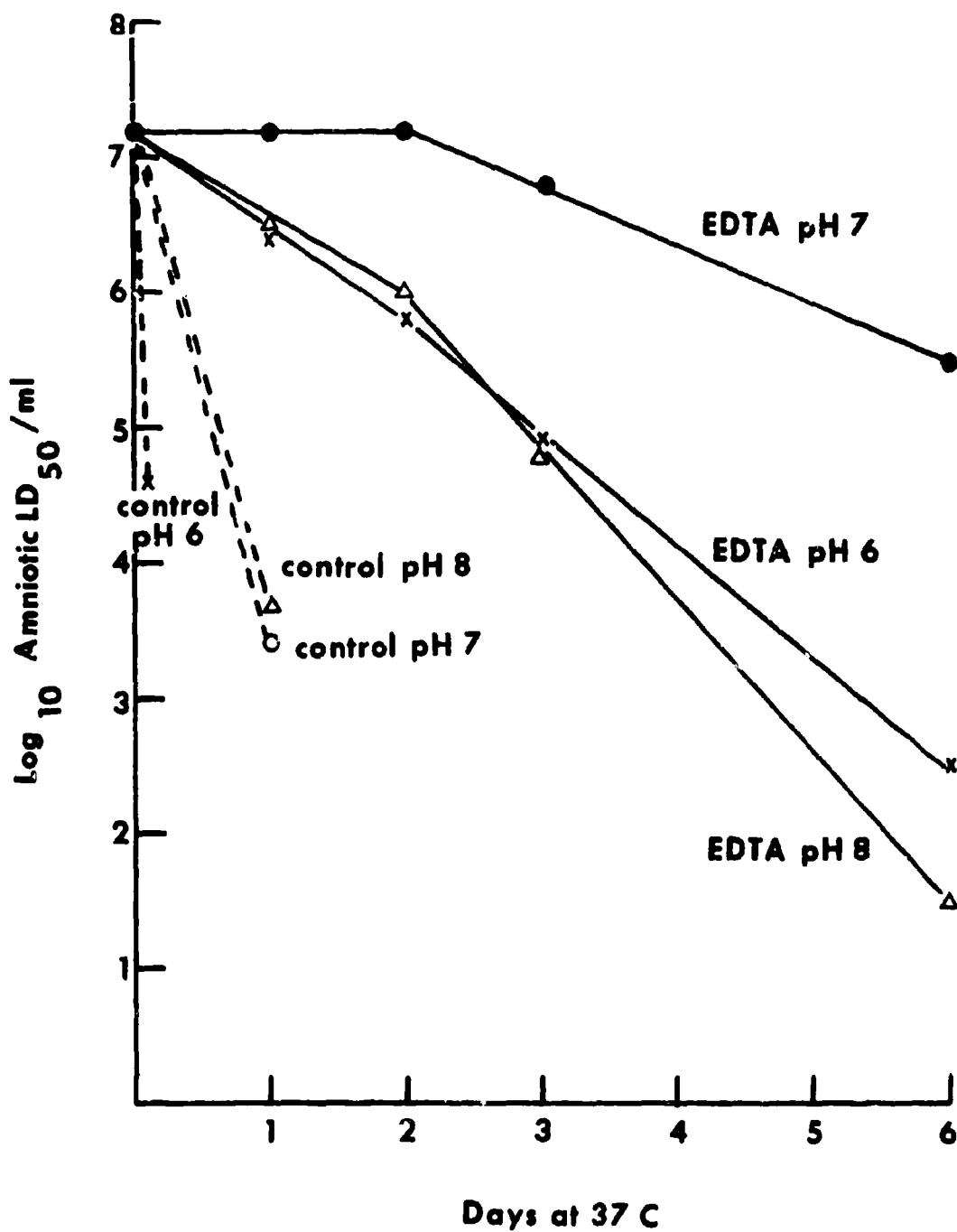


FIGURE 5. Effect of pH on the Stabilization of VEE Virus by EDTA at 37 C.

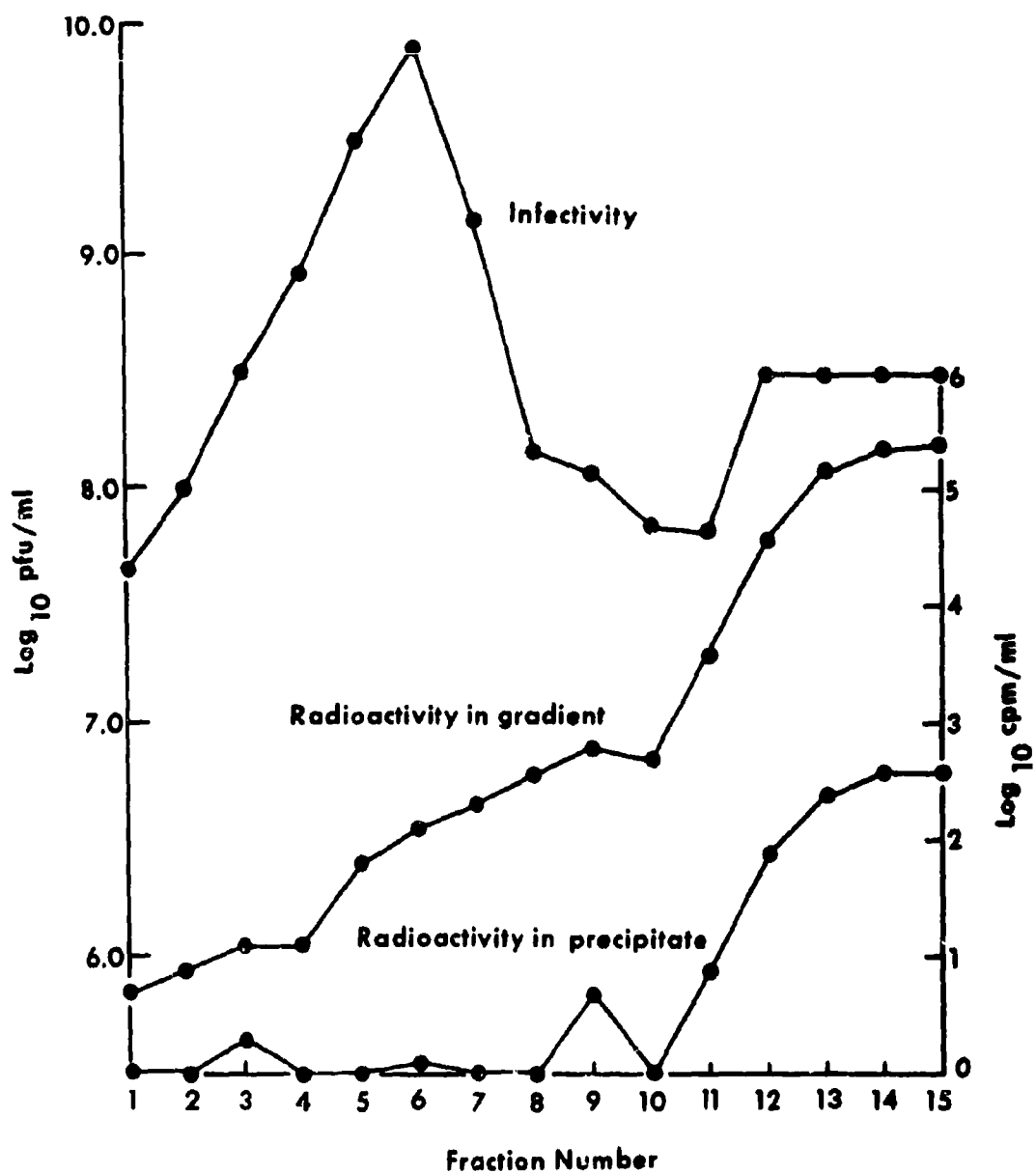


FIGURE 6. Sucrose Gradient Centrifugation of  $\text{C}^{14}$ -EDTA and VEE Virus Mixture.

Experiments have been conducted to determine whether the viral-stabilizing properties of EDTA would be eliminated or modified by competitive complex formation with metal ions. In a test at 25 C with calcium ion and partially purified VEE virus, using molar ratios of  $\text{Ca}^{++}$  to EDTA of 2:1, 1:1, and 0.5:1,  $\text{Ca}^{++}$  did not reduce the stabilizing capacity of EDTA during the 4-week duration of the test. The infectivity titers obtained are shown in Table 1. In similar tests with  $\text{Co}^{++}$  ion and EEE virus, a marked reduction in the stabilizing effect was observed, as indicated by the data in Table 2. Since cobalt itself was inactivating to the partially purified virus, titer decreases seen with the  $\text{Co}^{++}$  and EDTA combinations may reflect the inactivating character of  $\text{Co}^{++}$  rather than interference by  $\text{Co}^{++}$  with the stabilizing properties of EDTA. In a subsequent test with VEE virus and manganese ion (which was not inactivating to the virus),  $\text{Mn}^{++}$  reduced the stabilizing effect of EDTA, as shown by the data in Table 3. The results of the test at 25 C indicate that the  $\text{Mn}^{++}$  and EDTA complexes possessed some viral stabilizing capacity, but much less than EDTA alone. The differences observed with  $\text{Ca}^{++}$ ,  $\text{Co}^{++}$ , and  $\text{Mn}^{++}$  can be explained perhaps on the basis of differences in the equilibrium constants of the metal chelates formed, i.e., in the relative strengths of the complex of metal ion and EDTA. The constants for  $\text{Co}^{++}$  and  $\text{Mn}^{++}$  are higher,  $10^{16.31}$  and  $10^{14.04}$ , respectively, than for  $\text{Ca}^{++}$ ,  $10^{10.59}$ .

TABLE 1. EFFECT OF CALCIUM ION ON EDTA STABILIZATION OF VEE VIRUS

Suspending Medium Water Plus:		Infectivity, $\log_{10}$ amniotic $\text{LD}_{50}/\text{ml}$ , a/ after days at 25 C			
$\text{Ca}^{++}$	EDTA	2	6	22	29
0	0	6.6	4.5	<1.0	ND
0.005 M	0	6.8	6.2	<1.0	ND
0	0.005 M	7.3	6.8	6.8	5.8
0.01 M	0.005 M	7.4	6.9	6.6	6.4
0.005 M	0.005 M	7.5	6.9	6.4	6.0
0.0025 M	0.005 M	7.5	7.1	6.6	6.4

a. Starting titer:  $10^{7.5}$   $\text{LD}_{50}/\text{ml}$ .

TABLE 2. EFFECT OF COBALT ION ON EDTA STABILIZATION OF EEE VIRUS

Suspending Medium Water Plus:		Infectivity, $\log_{10}$ amniotic $LD_{50}/ml, \bar{x}/$ after days at 25 C		
$Co^{++}$	EDTA	1	2	3
0	0	7.6	5.5	<4.0
0.01 M	0	6.2	<4.0	<4.0
0	0.005 M	7.6	7.8	7.6
0.01 M	0.005 M	5.3	5.8	<3.0
0.005 M	0.005 M	5.4	<3.0	<2.0

a. Starting titer:  $10^{8.0} LD_{50}/ml$ .

TABLE 3. EFFECT OF MANGANESE ION ON EDTA STABILIZATION OF VEE VIRUS

Suspending Medium Water Plus:		Infectivity, $\log_{10}$ amniotic $LD_{50}/ml, \bar{x}/$ after days at 25 C				
$Mn^{++}$	EDTA	0.13	1	2	6	13
0	0	7.9	4.5	3.0	<1.0	ND
0.01 M	0	7.6	6.6	5.9	3.5	<1.0
0	0.005 M	7.3	7.6	7.8	7.2	6.4
0.01 M	0.005 M	7.2	7.2	6.9	5.4	<2.0
0.005 M	0.005 M	7.7	6.0	5.8	3.3	<2.0

a. Starting titer:  $10^{7.5} LD_{50}/ml$ .

Tests with EEE virus and Sindbis virus are not yet complete, but it appears that partially purified suspensions of these viruses are stabilized by EDTA to a degree similar to that of VEE virus.

#### IV. DISCUSSION

Because experiments with  $C^{14}$ -labeled EDTA did not indicate complex formation by EDTA with VEE virus, it seems likely that EDTA functions to protect viral infectivity by chelating traces of metal ions that may catalyze processes of inactivation, possibly oxidative in nature. This mode of action might be expected to apply to chelating agents in general. Tests to compare the relative stabilizing effects of EDTA and ammonium pyrrolidine dithiocarbamate (APDC), a very effective but nonspecific chelating agent, have shown that APDC enhances the thermal stability of VEE virus in a pH-dependent manner analogous to that observed with EDTA. Perhaps by employing chelating agents that are specific for different metal ions it may be possible to determine which metals are responsible for viral inactivation.

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13. ABSTRACT		
<p>Although a number of plant viruses are inactivated by ethylenediaminetetraacetic acid (EDTA), we have found that this chelating agent stabilizes Venezuelan equine encephalitis (VEE) virus and other group A arboviruses very effectively over the temperature range 4 to 50 C. In the presence of <math>5 \times 10^{-3}</math> M EDTA, the infectivity of partially purified VEE virus was maintained in demineralized water with no significant decrease for 3 days at 37 C, 2 to 4 weeks at 25 C, and 3 to 10 months at 4 C; at these temperatures control suspensions without EDTA lost 99.99% of their infectivity in 1 day, 2 to 4 days, and 10 to 30 days, respectively. These results were obtained with water or phosphate buffer suspensions only at the optimal pH range of 6.8 to 7.5. This degree of stabilization is comparable to that reported for poliovirus with 1 M <math>Mg^{++}</math>. Although the presence of EDTA is required for consistently successful phenol extraction of infectious RNA (IRNA) from partially purified VEE virus, thermal stabilization of IRNA by EDTA was not observed. Results obtained with density gradient centrifugation and other techniques using <math>C^{14}</math>-labeled EDTA suggest that EDTA does not form a stable complex with VEE virus; chelation of trace amounts of inactivating metal ions may constitute the mode of action.</p>		
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Venezuelan equine encephalitis virus	Infectivity	Cobalt ion
Eastern equine encephalitis virus	Stabilization	Manganese ion
Sindbis virus	Infectious ribonucleic acid	
Ethylenediaminetetraacetic acid	Chelating agents	
	Calcium ion	

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